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THE EFFECTS OF MULTIPLE INJECTIONS OF BACTERIAL ENDOTOXIN ON BLOOD COAGULATION IN THE TOADFISH, *OPSANUS TAU*

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Abstract

The effects of multiple injections of bacterial endotoxin on blood coagulation in the toadfish, *Opsanus tau,* were studied. Preparations of endotoxin from *Escherichia coli, Aeromonas hydrophila,* or *Vibrio harveyi* (strain 392) were utilized. Three injections of endotoxin were administered at either 6 or 12 h intervals.

Although there was a slight decrease in plasma fibrinogen concentration following the administration of endotoxin, levels did not significantly differ from normal controls. Endotoxins from *E. coli* and *A. hydrophila* initially shortened the prothrombin time (PT), but subsequently the PT returned to normal. In contrast, the partial thromboplastin time (PTT) was not shortened following the administration of bacterial endotoxin. There was no detectable effect on the plasma recalcification time. There were no significant differences between the red and white blood cell levels of control fish and fish that had received endotoxin. Endotoxins from *A. hydrophila* and *V. harveyi* reduced the survival of recipient toadfish, whereas *E. coli* did not increase mortality during the period of observation. Although both control and experimental fish demonstrated increased hematopoietic activity in the kidney, only *O. tau* that had received endotoxin demonstrated marked necrosis of interstitial cells.

Our data indicate that multiple injections of bacterial endotoxin into the toadfish result in alterations of blood coagulation compatible with activation of the blood coagulation mechanism. However, striking evidence of a consumptive coagulopathy, as is observed in mammals following multiple injections of bacterial endotoxin, was not observed. Interestingly, toadfish tolerated doses of endotoxin markedly greater than those sufficient to produce not only alterations of blood coagulation but also rapid death in many mammalian species.

Introduction

Blood coagulation has been studied in both healthy (Doolittle and Surgenor, 1962; Langdell *et al.,* 1965) and abnormal fish (Katz and Southward, 1950; Bouck and Ball, 1966; Hougie, 1971; Casillas *et al.,* 1975; Casillas and Smith, 1977). Coagulation disorders have been described in post-spawning Pacific salmon (Katz and Southward, 1950; Hougie, 1971) and in Pacific salmon following experimental stress (Bouck and Ball, 1966; Casillas and Smith, 1977). In fish subjected to experimental decompression, coagulation changes similar to those described in mammalian disseminated intravascular coagulation syndromes were observed (Casillas *et al.,* 1980).

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Impaired coagulation also has been observed in fish suffering from gram negative septicemia. Busch (1978) noted that the whole blood clotting time was markedly lengthened in trout infected with *Yersinia ruckeri,* a gram negative bacterium which causes enteric redmouth disease. Similarly, in fish infected with *Aeromonas salmonicida* recalcified plasma clots were more fragile than the normal clots (Field *et al.,* 1944), while blood from eels dying from *Vibrio* sp. infection coagulated poorly during the necropsies (McCarthy, 1976). Interestingly, Umbreit and Tripp (1975) demonstrated a heat stable toxin from *Vibrio anguillarum* which was lethal in goldfish. In addition to bacterial pathogens, prolonged whole blood clotting was observed in salmon infected with the virus responsible for viral hemorrhagic septicemia (Watson *et al.,* 1956).

Others have investigated a wide range of biological effects produced by endotoxin in fish, including lethality (Berczi *et al.,* 1966; Pol and Berg-Blommaert, 1980), antibody response (Paterson and Fryer, 1974; Ingram and Alexander, 1980), pyrogenicity (Reynolds *et al.,* 1977), hepatic glycogenolysis (Wedemeyer and Ross, 1968), and pituitary activation (Wedemeyer, 1969). *In vitro,* endotoxin was inactivated by fish serum (Von Eschen and Rudbach, 1974) and induced consumption of complement in fish serum (Day *et al.,* 1970). In contrast to mammals in which low doses of endotoxin produce pathologic effects, doses greater than 200 mg/kg have been required to produce lethality in fish (Berczi *et al.,* 1966) and intravenous injections as large as 47 mg/kg failed to affect the blood pressure (Wedemeyer and Ross, 1968). However, a lower dose (25 mg/kg) did cause a transient elevation in plasma cortisol six to eight hours post-injection (Wedemeyer, 1969). Following a much higher dose of *V. anguillarum* endotoxin (355 mg/kg), injected intraperitoneally, there was a significant decrease in plasma protein in coho salmon (Harbell *et al.,* 1979).

Although Hougie (1971) postulated that endotoxemia produced disseminated intravascular coagulation in diseased post-spawning salmon and a bacterial toxin was suspected to be active in eels dying from hemorrhagic septicemia due to a *Pseudomonas* species (Andre *et al.,* 1972), there has been no direct evidence linking endotoxin to abnormal blood coagulation in fish, as occurs in mammals (Levin and Beck, 1966). Furthermore, in addition to activating mammalian coagulation systems under certain conditions, multiple injections of endotoxin can produce renal lesions in association with thrombosis and hemorrhage (Bell *et al.,* 1972). Therefore, the following investigation was performed to identify possible effects of multiple injections of endotoxin on the blood coagulation system of fish and to detect related renal lesions. The saltwater teleost, *Opsanus tau,* was the test species. Three different preparations of endotoxin were evaluated: a commercially available lipopolysaccharide derived from *Escherichia coli;* a phenol extract of *Aeromonas hydrophila,* a freshwater fish pathogen; and a distilled water lysate of *Vibrio harveyi* (strain 392), a marine bacterium.

Materials and Methods

The toadfish, *Opsanus tau,* was used in all studies. Fish were selected from several hundred *O. tau* that had been captured 2–3 months previously and maintained in large concrete tanks equipped with flow through sea water, at the Marine Biological Laboratory supply department. Fish of approximately the same size (mean weight 168 g ± 6 g (1 SE)), showing normal behavior and no visible external lesions, were chosen for experimentation. The majority of the fish had not been fed since the time of capture and were not fed during the experiments, although in a few later experiments fish were selected from stocks that had had supplemental feeding with *Fundulus heteroclitus* for approximately two months. During the experiments, individual fish were identified by pieces of string inserted through the skin of either the dorsal or

pectoral fins and maintained in constantly flowing sea water within 5 gallon plastic buckets.

During either the injection or sampling procedures, the fish were restrained by wrapping them with wet cheesecloth and manually holding them down. To facilitate access to the gill arch vessels, into which intravenous injections were made and from which samples were taken, the two areas of loose skin connecting both the dorsal and ventral edges of the operculum to the body were incised, with no apparent harm to the fish. With the operculum reflected forward, the gill arches were well exposed. Plastic syringes (2.5 ml) and 26G needles were used for all injections or samples.

Preparations of endotoxin

Three types of endotoxin were used: a commercially available lipopolysaccharide prepared from *E. coli,* 026:B6 (Difco Laboratories, Inc., Detroit, Michigan); a phenol extract of *A. hydrophila;* and a crude lysate of *V. harveyi* in distilled water. The *A. hydrophila* (kindly provided by Dr. Alan Scott, Fisheries Department, Auburn University, Auburn, Alabama) was originally isolated from a moribund bluegill, *Lepomis macrochirus,* in a wild population of fish experiencing an epizootic. Phenol extraction of a 36 hour batch culture, grown in Brain Heart Infusion broth (Difco Laboratories, Inc.) at 30°C on a shaker table, was carried out according to the procedure outlined by Staub (1967).

The *V. harveyi* (strain 392), a common marine bacterium, was provided by Dr. Peter Greenberg, Department of Microbiology, Cornell University, Ithaca, New York. *V. harveyi* was grown for 6 hours in a batch culture in complete sea water media (5.0 g yeast extract, 3.0 g peptone, 3.0 g glycerol, 300 ml distilled water, and 700 ml sea water) (Nealson, 1978), at 30°C on a shaker table. After two washings with sterile 3% saline, the cells were boiled for 20 minutes in distilled water and allowed to remain at 4°C for an additional 2.5 hours. Following centrifugation, the supernatant was removed from the pelleted bacterial cells and used as a source of crude endotoxin. Prior to injection, sufficient sodium chloride was added to the distilled water lysate to yield a saline concentration of 0.9%.

Dose of endotoxins

When assayed by the Limulus amebocyte lysate test (Levin and Bang, 1968), the preparations of endotoxin from *A. hydrophila* and *V. harveyi* were equivalent to $\geq$100 mg/ml and 0.1 mg/ml of *E. coli* lipopolysaccharide (Difco Laboratories, Inc.), respectively. The total dose (based on biological activity in the Limulus test) of *V. harveyi* endotoxin varied from 0.004 to 0.008 mg/g of body weight, and of *A. hydrophila* from 2.4 to 4.4 mg/g body weight. A 0.1 mg/ml solution of *E. coli* was used, and the total dose ranged from 0.002 to 0.003 mg/g body weight. Control fish received injections of similar volumes of 0.9% saline.

All injections of *E. coli* and *A. hydrophila* endotoxins and the majority of *V. harveyi* endotoxin injections were administered at 12 hour intervals, designated (0, 12, 24 h). In a few experiments, *V. harveyi* endotoxin was injected at 6 hour intervals, designated (0, 6, 12 h). Control fish were injected using the appropriate schedule.

Blood samples

Since unlimited sampling was not possible due to the fixed number of gill arches, in most cases the initial blood sample was not obtained until after the second injection of endotoxin or saline. The majority of samples were obtained 6 or 12 hours after

an injection, depending upon the injection schedule. A control group of fish that had received no injections of either saline or endotoxin also was bled.

The volume of each sample of whole blood was measured in the syringe and after removal of the needle, the blood was expressed into a siliconized glass tube. Sufficient 3.8% sodium citrate was immediately added to produce a citrate:blood ratio of 1:9, the two were thoroughly mixed, and the tube was then placed in wet ice until the total blood cell count was completed. Subsequently, the sample was centrifuged for 10 minutes at 2000 rpm in a table top centrifuge and the plasma then removed from the red blood cells and buffy coat with a pasteur pipet. The plasma sample was either placed in wet ice or held at 4°C in a refrigerator until tests of plasma coagulation could be completed. Although the tests were not affected by storage at 4°C for 48 hours, all determinations were completed within 24 hours after the sample was obtained. Frozen samples were unsuitable and were never used.

Mortality

All mortalities were recorded as hours to death after the initial injection of endotoxin. In several cases, fish were sacrificed *in extremis*. However, some fish died unobserved overnight. In the latter cases, the time of death was estimated as closely as possible, based on the condition of the carcass.

Histopathological analysis

Necropsies were performed on all fish and representative samples of kidney and spleen were removed and fixed with 10% buffered formalin. Following fixation, the tissues were embedded in paraffin; 6 micron sections were prepared and stained with hematoxylin and eosin by standard methods. All tissue sections were examined with light microscopy.

Red and white blood cell counts

The number of both red and white blood cells was determined by a method similar to that described by Klontz and Smith (1968). Briefly, citrated whole blood was diluted 1/200 with Rees-Ecker counting fluid in a red blood cell diluting pipet and allowed to stand for approximately 10 minutes at room temperature. Both chambers of a modified Neubauer hemocytometer were filled from one sample and the counts of the two chambers were averaged. Under 400× magnification, erythrocytes appeared as elliptical cells with an elliptical nucleus and abundant clear to pale blue cytoplasm; while white blood cells were smaller, round, dark blue staining cells with little or no apparent cytoplasm. It was impossible to distinguish thrombocytes from other white blood cells.

Tests of blood coagulation

A small sample (approximately 0.4 ml) of unanticoagulated whole blood was placed in a clean glass tube in order to determine the whole blood clotting time. The tube was gently tilted every 5 to 10 minutes to observe any clot formation. The appearance and time of formation of the clot were noted and the tube was then covered and set aside for later observation of clot retraction.

The fibrinogen concentration in citrated plasma was determined by the serial dilution technique outlined by Hougie (1977). Serial twofold dilutions of plasma (to

1:128) were prepared in 0.14 *M* phosphate buffered saline (PBS), pH 7.2. An equal volume of a solution of bovine thrombin (Sigma Chemical Company, St. Louis, Missouri) containing approximately 1 NIH unit of thrombin in PBS was added to each dilution (final concentration of thrombin in assay, 5 U/ml). Approximately 90–120 minutes after the addition of thrombin, the tubes were checked for the presence of a gel by gently tipping the tube. After the highest dilution containing a gel was recorded, the tubes were covered and allowed to sit at room temperature until the next morning, when the highest dilution containing a gel was recorded again.

Hougie's method for measuring the prothrombin time was used with some slight modifications (Hougie, 1971). Brains of *O. tau,* freed of overlaying meninges and blood vessels, were ground up in PBS. The suspension was placed in a refrigerator at 4°C for 1 hour to allow the largest pieces of brain tissue to settle. The supernatant was then removed and used as a complete thromboplastin, after the dilution of thromboplastin that produced the shortest prothrombin time was determined. Small samples of the complete thromboplastin, twice as concentrated as the optimal dilution, were stored at −20°C and reconstituted with PBS prior to use. The prothrombin time test was performed by mixing equal volumes (0.05 ml) of citrated plasma and complete thromboplastin in a clean glass tube, allowing the mixture to incubate for 30 seconds at room temperature, and then adding 0.05 ml of 0.025 *M* $CaCl_2$. The tube was tilted by hand with a regular motion and the time required for gel formation was measured to the nearest second with a stopwatch.

A partial thromboplastin was extracted from cleaned *O. tau* brains according to the method described by Rapaport *et al.* (1954) and was standardized and stored in the same fashion as the complete thromboplastin. The partial thromboplastin time test was performed according to the method of Goulian and Beck (1965) with only slight modification. The kaolin suspension (10 mg/ml) was added to the partial thromboplastin in the assay tube just prior to the test (0.05 ml of each reagent). Citrated plasma (0.05 ml) was then added to the mixture and allowed to incubate for 30 seconds at room temperature. Following incubation, 0.05 ml of 0.045 *M* $CaCl_2$ was added and the time required for gel formation was measured to nearest second with a stopwatch.

The recalcification time of citrated plasma was measured by mixing equal volumes of undiluted plasma and 0.025 *M* $CaCl_2$ in a clean glass tube. Ordinarily, several plasma samples were assayed simultaneously rather than each sample separately. Therefore, the intervals between observations of one tube were occasionally as long as 30 seconds. Each tube was gently tilted sequentially until a gel was detected. For all coagulation tests, the entire procedures were performed at room temperature (25°).

All data were analyzed by the Student *t*-test.

Results

Survival after endotoxin administration

Injection of *V. harveyi* endotoxin (0, 12, 24 h schedule) caused a significant decrease ($P < 0.05$) in the mean survival time in comparison with saline injected fish (Fig. 1). Furthermore, *V. harveyi* (0, 6, 12 h schedule) significantly decreased the mean survival time in comparison with the other injection schedule of *V. harveyi* (0, 12, 24 h) ($P < 0.05$). Although differences in survival between the different types of endotoxin were not significant, *V. harveyi* and *A. hydrophila* endotoxins appeared more lethal than did *E. coli* endotoxin.

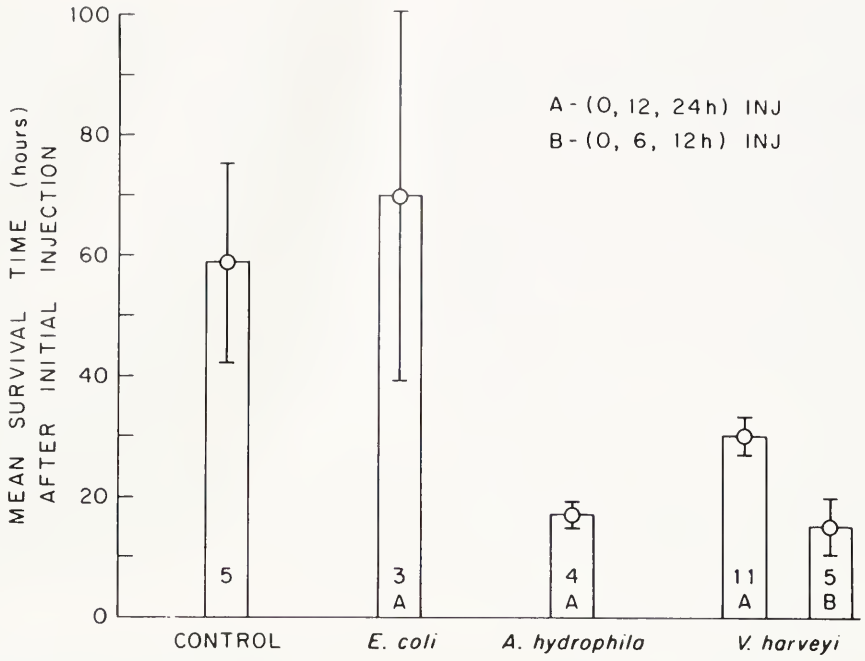


FIGURE 1. *Survival after endotoxin administration.* Bars indicate the mean survival time ± 1 standard error of the mean (S.E.M.) following the initial injection of endotoxin. The control data from both of the injection schedules (0, 12, 24 h and 0, 6, 12 h) did not differ significantly and therefore were combined. Injection schedules: A = (0, 12, 24 h) and B = (0, 6, 12 h). Number of fish in each sample is indicated in each bar. The type of endotoxin is designated beneath each bar.

Plasma fibrinogen concentration

The mean plasma fibrinogen titer, *i.e.,* the highest dilution of plasma that formed a detectable gel after addition of thrombin, of normal unbled *O. tau* was 1:32 (Fig. 2). After three injections of 0.9% NaCl (approximately 10 ml total dose) and removal of a total of approximately 4.0 ml of blood in two earlier blood samples, the fibrinogen concentration fell only insignificantly (Fig. 2). A decrease after repeated injections and removal of samples also occurred in animals injected with endotoxin, although the reductions of fibrinogen in endotoxin injected fish were not significantly lower than corresponding control fish. However, the fibrinogen concentration after only one injection of *E. coli* endotoxin was significantly greater ($P < 0.01$) than in uninjected control fish. Subsequently, in the fish injected with *E. coli* endotoxin, the concentration of fibrinogen in the post-third injection sample was significantly decreased ($P < 0.05$) from the post-first injection sample (Fig. 2).

Prothrombin time

The prothrombin time (PT) of uninjected control fish was approximately 33 seconds and shortened slightly after animals had received three injections of 0.9% NaCl and two blood samples had been taken (Fig. 3). In comparison with saline injected animals, one injection of *A. hydrophila* or *E. coli* endotoxin caused the PT to shorten significantly ($P < 0.01$) or insignificantly, respectively. Following two more

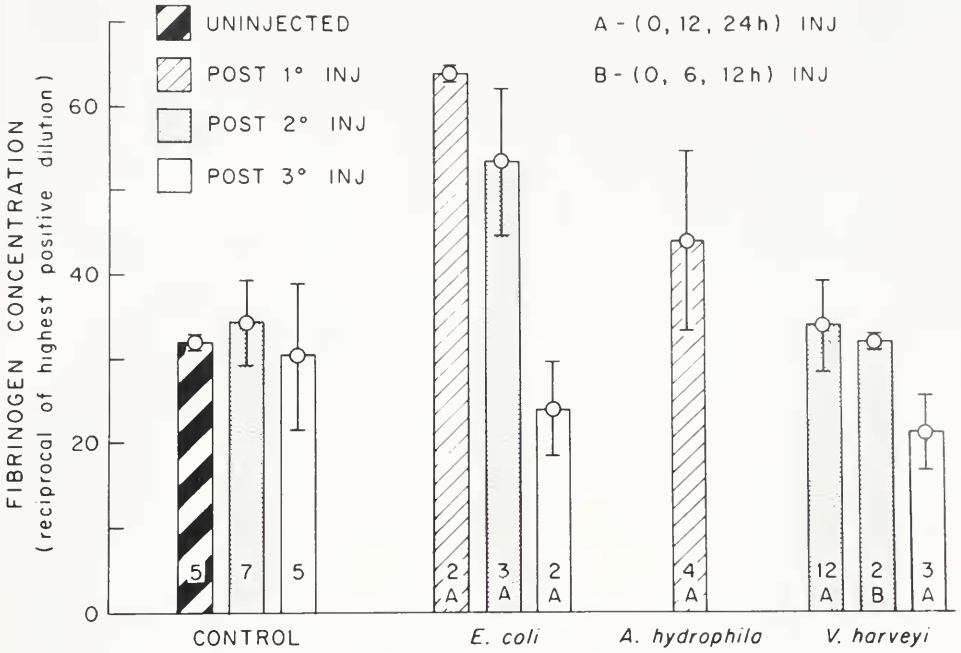


FIGURE 2. *Plasma fibrinogen concentration.* Bars indicate the reciprocal ± 1 S.E.M. of the highest plasma dilution that formed a visible gel following the addition of bovine thrombin (final concentration, 5 U/ml). The control data from both of the injection schedules (0, 12, 24 h and 0, 6, 12 h) did not differ significantly and therefore were combined. Endotoxin injection schedules: A = (0, 12, 24 h) and B = (0, 6, 12 h). Number of fish in each sample is indicated in each bar. Heavily striped bar = uninjected controls. Lightly striped bar = post-first injection. Stipled bar = post-second injection. Open bar = post-third injection. The type of endotoxin is indicated beneath each cluster of bars. Initially uninjected controls were used for statistical comparison with post-first injection samples from experimental animals to minimize the number of samples required from saline injected control animals. Additional samples were obtained from controls after the second and third injections of saline.

injections of *E. coli* endotoxin, the PT lengthened to comparable control levels. *V. harveyi* endotoxin did not significantly change the PT in comparison with saline injected animals, nor was there a significant difference between injection schedules.

Interestingly, in preliminary experiments, complete thromboplastin (brain extract in 0.9% NaCl) prepared from the red hake, *Urophycis chuss,* also a marine fish, resulted in a longer PT than did the homologous thromboplastin prepared from *O. tau* brains.

Partial thromboplastin time

The partial thromboplastin time (PTT) of normal control animals was approximately 55 seconds, and in contrast to the PT, became prolonged, though not significantly, following multiple injections of saline and removal of blood samples (Fig. 4). No significant differences were observed between endotoxin and saline injected animals. In *E. coli* endotoxin injected animals, there was an increase in PTT between the post-second injection sample and the post-third injection sample, although the increase was not significant.

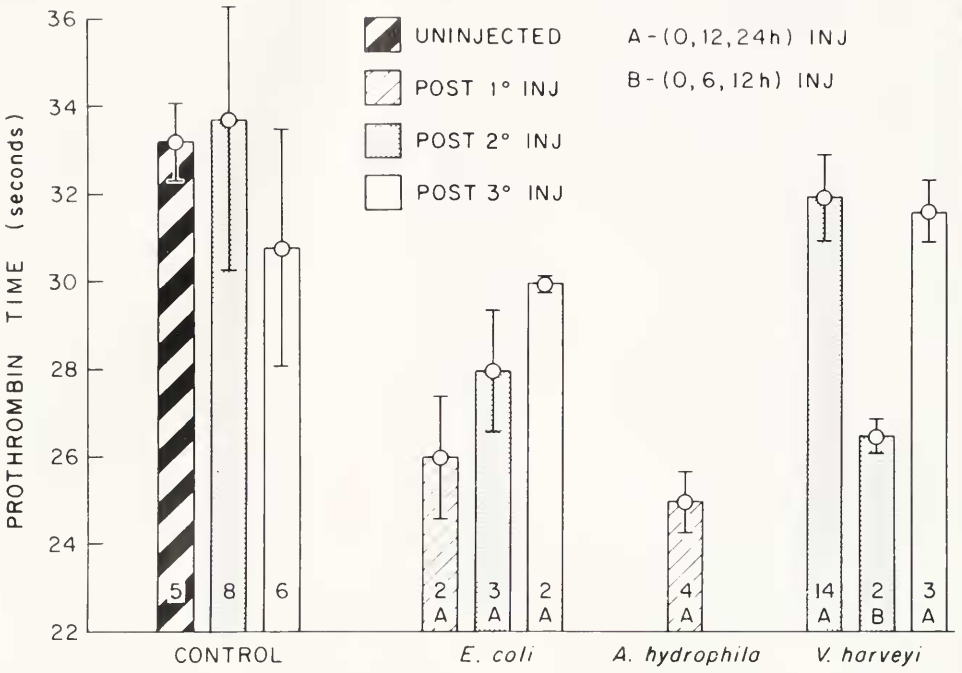


FIGURE 3. *Prothrombin time.* Bars indicate the mean time in seconds ± 1 S.E.M. for gel formation to occur following the addition of Ca^{+2} to an equivolume mixture of plasma and saline brain extract (See Methods). The control data from both of the injection schedules (0, 12, 24 h and 0, 6, 12 h) did not differ significantly and therefore were combined. Endotoxin injection schedules: A = (0, 12, 24 h) and B = (0, 6, 12 h). Number of fish in each sample is indicated in each bar. Heavily striped bar = uninjected controls. Lightly striped bar = post-first injection. Stipled bar = post-second injection. Open bar = post-third injection. The type of endotoxin is indicated beneath each cluster of bars.

Whole blood coagulation time

Due to procedural difficulties, it was impossible to obtain and observe sufficient numbers of samples for quantitative and statistical analysis. However, there was a clear trend in both experimental and control animals towards a shortening of the whole blood coagulation time (WBCT) following the initial blood sample. Although in all groups of fish the WBCT in the first sample was highly variable and frequently greater than 2 hours, (mean, 2.3 ± 0.5 h), the WBCT in the second sample was in all cases less than 40 minutes and in two thirds of the second samples the blood coagulated within 20 minutes. In no animals did the WBCT lengthen with repeated sampling.

In addition to changes in the coagulation time, the appearance of the clot also altered. When the coagulation time in the first sample was lengthened, *i.e.,* hours rather than minutes elapsed until coagulation, the clot that eventually formed was only semi-solid and did not involve the entire volume of the sample. However, in later samples when the coagulation time shortened, the clot became more solid and usually involved the entire sample. Due to the small size of the sample and the poor quality of the clots, it was often impossible to evaluate the degree of clot retraction that had occurred. However, clot retraction was observed in those samples in which solid clots formed initially.

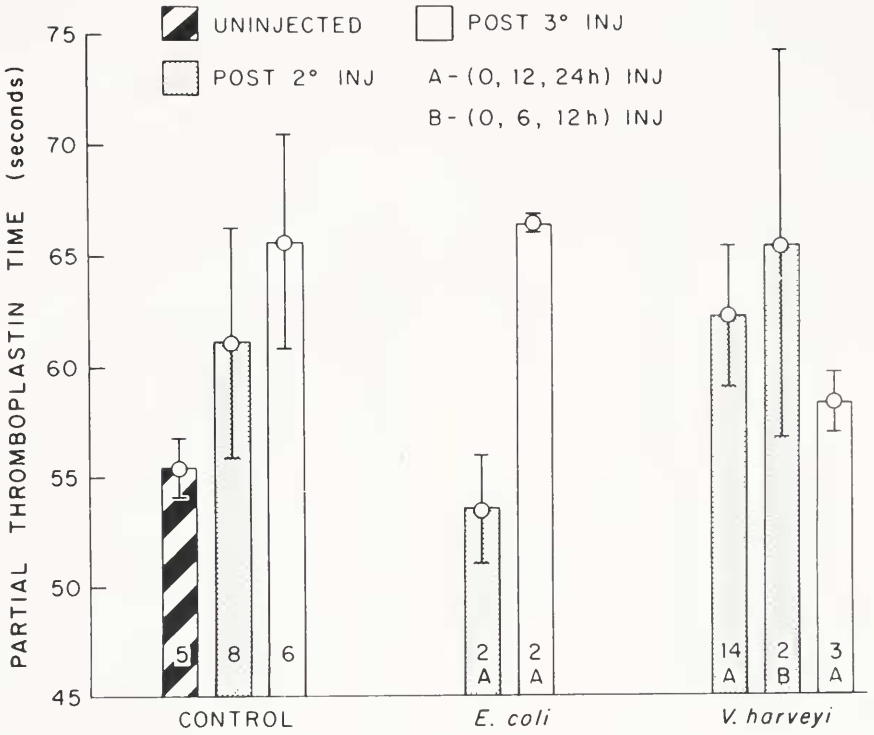


FIGURE 4. *Partial thromboplastin time.* Bars indicate the mean time in seconds ± 1 S.E.M. for gel formation to occur following addition of Ca^{+2} to an equivolume mixture of plasma, partial thromboplastin, and kaolin suspension (See Methods). The control data from both of the injection schedules (0, 12, 24 h and 0, 6, 12 h) did not differ significantly and therefore were combined. Endotoxin injection schedules: A = (0, 12, 24 h) and B = (0, 6, 12 h). Number of fish in each sample is indicated in each bar. Heavily striped bar = uninjected controls. Stipled bar = post-second injection. Open bar = post-third injection. The type of endotoxin is indicated beneath each cluster of bars.

Plasma recalcification time

The plasma recalcification time was variable in both normal and experimental animals with a range of 1 to 8 minutes; and the endpoint was not well defined because coagulation generally occurred during a 30 to 60 second period. Therefore, the recalcification time was difficult to evaluate accurately. All samples did eventually form a gel, with the exception of one uninjected control that failed to clot even after 15 minutes of observation. From the available observations there appeared to be no effect of endotoxin on the recalcification times.

Red blood cell count

The red blood cell count declined significantly ($P < 0.01$) from a normal level in uninjected fish of 4.6×10^5 erythrocytes/mm^3 to approximately 1.4×10^5 erythrocytes/mm^3 following three injections of saline and two blood samples (Fig. 5). Similarly, animals injected with *V. harveyi* (0, 12, 24 h) showed a significant decline ($P < 0.05$) in the number of red blood cells between the post-second injection and post-third injection samples. There were, however, no significant differences between any of the endotoxin and comparable saline injected animals.

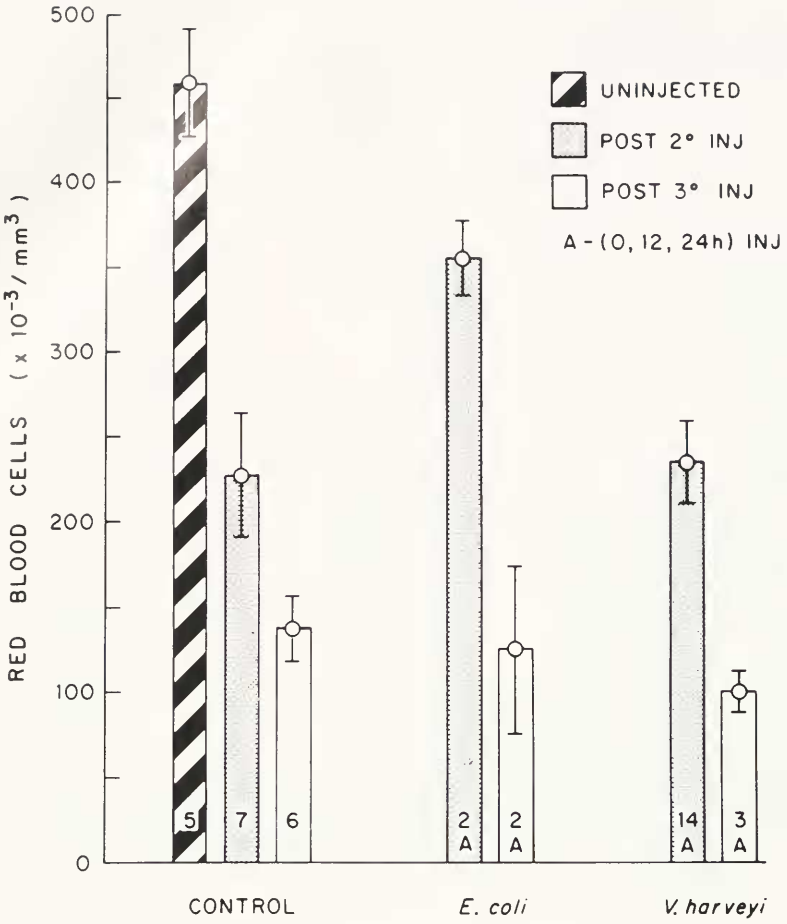


FIGURE 5. *Red blood cell count.* Bars indicate the mean number ± 1 S.E.M. of erythrocytes/mm^3. The control data from both of the injection schedules (0, 12, 24 h and 0, 6, 12 h) did not differ significantly and therefore were combined. Endotoxin injection schedules: A = (0, 12, 24 h). Number of fish in each sample is indicated in each bar. Heavily striped bar = uninjected controls. Stipled bar = post-second injection. Open bar = post-third injection. The type of endotoxin is indicated beneath each cluster of bars.

White blood cell count

As in the red blood cell counts, there was a general decline in white blood cell numbers in the post-third injection samples for all treatments (Fig. 6). However, this decline was not significant in contrast to the decreases observed in red blood cell numbers.

The number of white blood cells was significantly less ($P < 0.01$) in the *V. harveyi* endotoxin (0, 12, 24 h) injected animals than in saline injected animals, in the post-second injection samples (Fig. 6). By the post-third injection samples, there was no difference between saline and *V harveyi* injected animals, although the white blood cell count in the *V. harveyi* injected animals had continued to decline.

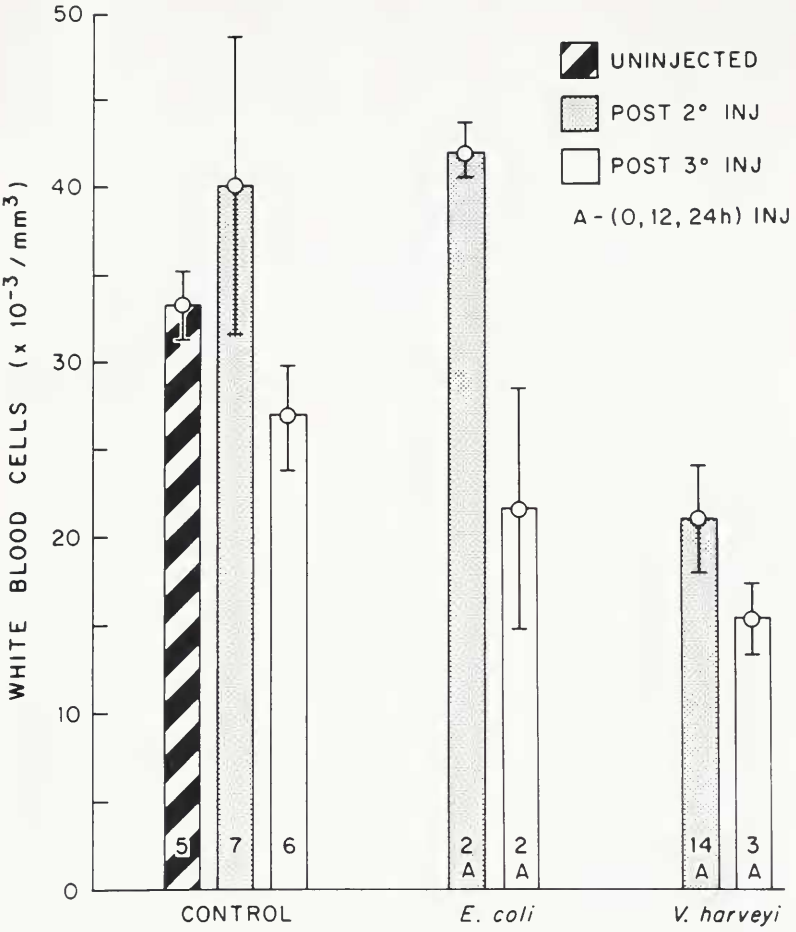
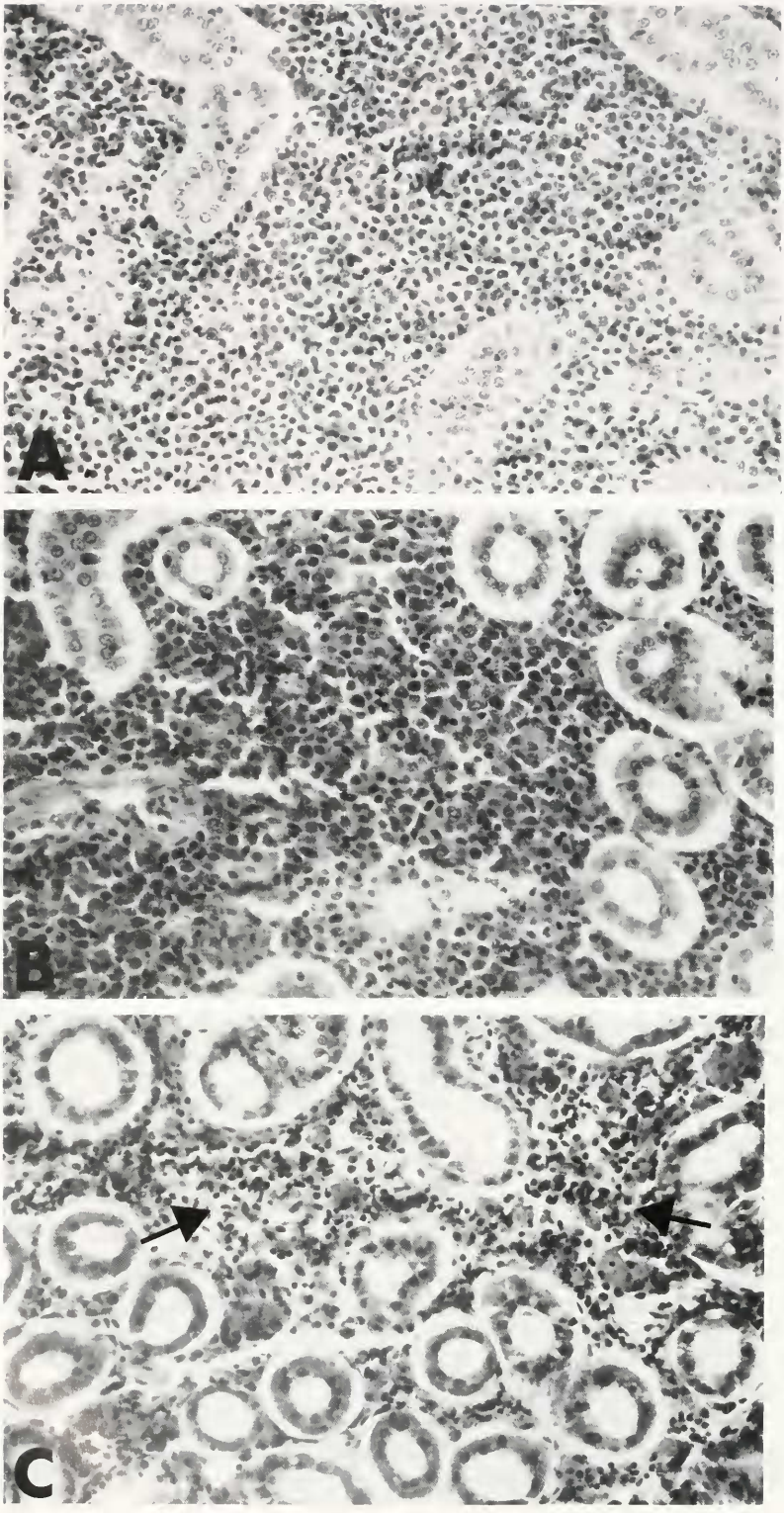


FIGURE 6. *White blood cell count.* Bars indicate the mean number ± 1 S.E.M. of white blood cells/ mm^3. The control data from both of the injection schedules (0, 12, 24 h and 0, 6, 12 h) did not differ significantly and therefore were combined. Endotoxin injection schedules: A = (0, 12, 24 h). Number of fish in each sample is indicated in each bar. Heavily striped bar = uninjected controls. Stipled bar = post-second injection. Open bar = post-third injection. The type of endotoxin is indicated beneath each cluster of bars.

Clinical observations

On several occasions, it was impossible to obtain a blood sample from an animal due to apparent stasis of blood within the branchial vessel, as evidenced by a lack of hemorrhaging following withdrawal of the needle. Prior to these unsuccessful sampling attempts, the fish were unresponsive to stimuli, and succumbed shortly after being returned to water.

After injections of either endotoxin or saline, many fish demonstrated a period of abnormal behavior, which lasted up to 30 minutes and was characterized by alternating periods of frenzied swimming and extreme lethargy. During the latter


A
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part of the injection schedule, the periods of lethargy were more pronounced and lengthened. Furthermore, in many instances a transient change from a normal dark brown color to a pale yellowish-brown color accompanied this period of abnormal behavior.

Histological examination

The primary change observed in the kidneys was a loss of normal interstitial architecture due to a combination of cellular necrosis and compensatory hematopoietic activity. Generally, kidneys of saline injected fish displayed hematopoietic activity in the absence of cellular necrosis, whereas the kidneys of endotoxin injected fish, paticularly those injected with *V. harveyi* endotoxin, demonstrated marked cellular necrosis in addition to compensatory hematopoietic activity (Fig. 7). The renal interstitial tissue of an uninjected fish (Fig. 7a) or a saline injected fish (Fig. 7b) appears intact and contains a normal amount of hematopoietic cells. However, following injection of *V. harveyi* lysate, there was a marked loss of hematopoietic tissue due to severe necrosis (Fig. 7c). Although in this fish the necrosis was severe, other fish injected with endotoxin occasionally showed similar, but less marked lesions. Tubular necrosis was also occasionally observed. Since *O. tau* is aglomerular, it was impossible to observe glomerular fibrin deposition as is observed in the generalized Shwartzman reaction. No histological differences between groups were noted in sections of spleens.

Discussion

Blood coagulation was evaluated in the toadfish, *Opsanus tau,* following multiple injections of endotoxin, and although differences between saline and endotoxin injected animals were not necessarily consistent with a consumption coagulopathy in all cases, there were several significant changes related to the injection of endotoxin. The most obvious effect of repeated injections and removal of blood samples was the development of anemia in all fish, both experimental and control. Assuming that a blood volume of approximately 3% of body weight for marine fish, as reported by Thorson (1961), applies to *O. tau,* each sample of 1.5 to 2.0 ml removed up to 30% of the total blood volume. This large blood loss was undoubtedly a significant stress and accounts for the observed anemia; and furthermore must be considered when other hematological and histopathological changes are interpreted.

Although high doses of endotoxin caused increased mortality, there was no correlation between lethality and dose of endotoxin as measured by the Limulus amebocyte lysate assay. *V. harveyi* endotoxin was more lethal than approximately the same total dose of *E. coli* endotoxin. Furthermore, *A. hydrophila* endotoxin, which was injected in much greater amounts ranging from 2.4 to 4.4 mg/g, was no more lethal than *V. harveyi* endotoxin. Lack of correlation between the dose and lethality may have resulted in part from the different sources of endotoxin and their manner of preparation. In any case, the amount of endotoxin injected was far in excess of the dose required to perturb mammalian systems. However, we are unaware of any report in which endotoxin levels were measured in the blood of fish during gram negative septicemia,

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FIGURE 7. *Photomicrographs of posterior kidney.* Normal interstitial hematopoietic tissue in the posterior kidney of uninjected fish (7A) and fish injected three times with saline (7B) is compared with abnormal posterior kidney of fish injected two times with *V. harveyi* lysate (7C). Note the areas of necrosis and loss of interstitial cellularity (indicated by arrows) in fish after injection of *V. harveyi* lysate (7C). (Hematoxylin-eosin stain; original magnification ×100).

although a severe bacteremia of 10^6 viable bacteria/ml of blood has been reported in eels experimentally infected with *Aeromonas liquefaciens* (Hatai, 1972).

The clinically observed period of abnormal behavior following injections represents a non-specific stress response. Pigmentation changes also often occur in response to general stress and are frequently detected during bacterial infections (Richards and Roberts, 1978).

The observation of blood stasis in the branchial vessels of moribund fish may be in part explained by hypercoagulability of the blood. When a sample was drawn from such an animal, the blood invariably coagulated within a minute. Hypercoagulability may have been due to local tissue damage following multiple samples and injections in addition to the direct effects of bacterial endotoxin. The removal of a substantial volume of blood also probably caused decreased blood pressure which further contributed to the stasis of blood.

At the post-second injection sample, *V. harveyi* endotoxin injected animals had significantly lower leukocyte counts than comparable saline injected animals. It is tempting to postulate that the decrease in leukocytes is analogous to the initial decrease in white blood cell levels observed in mammals injected with endotoxin, although in mammals leukopenia occurs much sooner after injection of endotoxin. However, the ultimate decline in the leukocyte number in the final samples obtained from all fish was probably produced, at least in part, by the same factors that caused the aforementioned anemia. Interestingly, the reduction in leukocytes was not nearly as severe as the decline in erythrocytes.

Inasmuch as the injection and sampling schedule caused a decline in the number of circulating blood cells, one might also have observed a lengthening of the plasma coagulation tests due to the physical removal of essential coagulation factors. In some tests, however, there was a shortening rather than a lengthening of the coagulation times. The whole blood coagulation time (WBCT) clearly showed this trend. Not only did the clot form more quickly in later samples, but the quality of the clot was better, indicating that if there was a diminution of some clotting factors, it did not affect the rate of coagulation.

The normal fibrinogen titer of *O. tau* was within the normal ranges of human (Hougie, 1977) and salmonid (Casillas *et al.*, 1975) fibrinogen concentrations. The insignificant decreases in fibrinogen levels in the post-third injection samples in both saline and *V. harveyi* endotoxin injected animals may have resulted from repeated sampling. However, as indicated by the WBCT and plasma coagulation tests, there was still a sufficient concentration of fibrinogen available for clot formation. There is no clear explanation for the observed elevated fibrinogen level following the initial injection of *E. coli* endotoxin, though it may represent early hypercoagulability. In mammals, elevated fibrinogen levels have been observed in both clinical states (Sack *et al.*, 1977) and experimental models (Cooper *et al.*, 1971) associated with disseminated intravascular coagulation.

The prothrombin time (PT) measured in *O. tau* was markedly longer than values reported for salmonids (Hougie, 1971; Casillas *et al.*, 1975). In comparison to salmon, *O. tau* is extremely sluggish and may not require a rapidly responsive coagulation system. Additionally, the PT appeared unresponsive to repeated sampling and injections, since the control times remained fairly constant in all samples (there was an insignificant shortening in the post-third injection sample). It is noteworthy that in preliminary PT tests, the use of a saline brain extract from the red hake, *Urophycis chuss*, rather than the extract from *O. tau*, resulted in markedly lengthened PT times. While the effects of different thromboplastins have been studied with fish, comparisons

were made between brain tissues from different classes of animals (Doolittle, 1962; Hougie, 1971). However, our preliminary results indicate that there appear to be differences in fish thromboplastins obtained from separate species.

Although the PT has been reported to be prolonged in consumptive coagulopathy syndromes (Colman *et al.,* 1972), prolongation has not been uniformly detected. However, both *E. coli* and *A. hydrophila* endotoxins appeared to affect the coagulation system of *O. tau,* causing a shortening of the PT after the initial injection. In *E. coli* endotoxin, this initial shortening was followed by a steady lengthening of the PT, although it never became elevated above control values.

The effect of *V. harveyi* endotoxin on the PT is difficult to explain. While two injections of the endotoxin administered at 12 hour intervals had no apparent effect on the PT, two injections given at 6 hour intervals caused an apparent decrease in the PT. Possibly, the two injections of *V. harveyi* endotoxin at 6 hour intervals were close enough together to produce a level of endotoxemia sufficient to cause a hypercoagulable state similar to that observed after one injection of *E. coli* or *A. hydrophila.* In contrast, injections at 12 hour intervals may have allowed sufficient time for the endotoxin to be cleared from the circulation after the first injection.

When compared with values reported in salmonids (Hougie, 1971; Casillas *et al.,* 1975), the partial thromboplastin time (PTT) in *O. tau* was longer, though to a lesser extent than that observed in the PT. Unlike the PT, the PTT increased in both saline and endotoxin injected animals, following multiple injections and samples. Whether these two different responses to the same stimuli by the coagulation system reflect physiologically significant differences between extrinsic and intrinsic coagulation systems in *O. tau* is unclear. Our data suggest that both systems are present and that repeated sampling can more easily deplete some factor(s) involved with intrinsic coagulation in *O. tau.*

The recalcification time (Recal time) of *O. tau* was longer than the Recal time of the tautog, *Tautoga onitis,* a saltwater fish (Doolittle and Surgenor, 1962), and failed to demonstrate a good endpoint. The Recal time, like the WBCT, is dependent on the plasma concentrations of many coagulation factors and therefore is a good indicator of the overall function of the coagulation system. However, unlike the WBCT, which showed dramatic decreases particularly just prior to death, there appeared to be no comparable shortening of the Recal time. A partial explanation for this difference may be that activated thrombocytes were present in the whole blood but were absent from the samples of plasma. The contribution of thrombocytes to fish coagulation has been reported previously (Katz and Southward, 1950; Doolittle and Surgenor, 1962).

The results of histological examinations are somewhat difficult to interpret. Since the interstitial tissue of fish is the primary hematopoietic organ the lesions in the interstitial tissue could not be definitely linked with the injection of endotoxin. Due to the experimentally induced anemia, histological changes characteristic of compensatory hematopoietic activity, unrelated to the endotoxin injections, occurred to some degree in all fish. Nevertheless, injections of endotoxin appeared to cause more severe lesions, characterized by cellular necrosis in the interstitial tissue. Since endotoxin injected animals demonstrated increased mortality, these lesions are likely to be pathological.

As noted earlier, *O. tau* is a particularly sluggish fish and this may account, in part, for the lack of response of its blood coagulation system to endotoxin. In recent studies of rainbow trout, a highly active fish, changes in the coagulation system similar to disseminated intravascular coagulation were measured during experimental *Y.*

ruckeri infections (Miller, 1982; Miller, 1983). Furthermore, preliminary evidence indicated that intracardial injection of *Y. ruckeri* endotoxin caused a similar condition. These marked differences between species of fish warrant further consideration.

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LITERATURE CITED

Andre, P. G., D. A. Conroy, D. McGregor, R. J. Roberts, and H. Young. 1972. Acute hemorrhagic septicemia in captive european eels (*Anguilla vulgaris*). A clinical and pathological study. *Vet. Rec.* **90:** 726–729.

Bell, W. R., R. E. Miller, and J. Levin. 1972. Inhibition of the generalized Shwartzman reaction by hypofibrinogenemia. *Blood* **40:** 697–708.

Berczi, I., L. Bertok, and T. Bereznai. 1966. Comparative studies on the toxicity of *E. coli* lipopolysaccharide endotoxin in various animal species. *Can. J. Microbiol.* **12:** 1070–1071.

Bouck, G. R., and R. C. Ball. 1966. Influence of capture methods on blood characteristics and mortality in rainbow trout (*Salmo gairdneri*). *Trans. Am. Fish. Soc.* **95:** 170–176.

Busch, R. A. 1978. Enteric redmouth disease (Hagerman strain). *Mar. Fish. Rev.* **40:** 42–51.

Casillas, E., and L. S. Smith. 1977. Effect of stress on blood coagulation and hematology in rainbow trout (*Salmo gairdneri*). *J. Fish Biol.* **10:** 481–491.

Casillas, E., L. S. Smith, J. J. Woodward, and B. G. D'Aoust. 1980. Hematologic response of fish to changes in gas or hydrostatic pressure. *Am. J. Physiol.* **239:** R161–R167.

Casillas, E., S. E. Miller, L. S. Smith, and B. G. D'Aoust. 1975. Changes in hemostatic parameters in fish following rapid decompression. *Undersea Biomed. Res.* **2:** 267–276.

Colman, R. W., S. J. Robboy, and J. D. Minna. 1972. Disseminated intravascular coagulation (DIC): an approach. *Am. J. Med.* **52:** 679–689.

Cooper, H. A., E. J. W. Bowie, P. Didisheim, and C. A. Owen, Jr. 1971. Paradoxic changes in platelets and fibrinogen in chronically induced intravascular coagulation. *Mayo Clin. Proc.* **46:** 521–523.

Day, N. K., R. A. Good, J. Finstad, R. Johannsen, R. J. Pickering, and H. Gewurz. 1970. Interactions between endotoxic lipopolysaccharides and the complement system in the sera of lower vertebrates. *Proc. Soc. Exp. Biol. Med.* **133:** 1397–1401.

Doolittle, R. F., and D. M. Surgenor. 1962. Blood coagulation in fish. *Am. J. Physiol.* **203:** 964–970.

Field, J. B., L. L. Gee, C. A. Elvehjem, and C. Juday. 1944. The blood picture in furunculosis induced by bacterium salmonicida. *Arch. Biochem.* **3:** 277–284.

Goulian, M., and W. S. Beck. 1965. The partial thromboplastin time test. Modification of the procedure, and study of the sensitivity and optimal conditions. *Am. J. Clin. Pathol.* **44:** 97–103.

Harbell, S. L., H. O. Hodgins, and M. H. Schiewe. 1979. Studies on the pathogenesis of vibriosis in coho salmon *Oncorhynchus kisutch* (Walbaum). *J. Fish Dis.* **2:** 391–404.

Hatai, K. 1972. Studies on the fate of intravascularly injected bacteria in fish. I. Changes in number of cells of *Aeromonas liquefaciens* in blood, liver, and kidneys of the eel. *Fish Pathol.* **7:** 26–33.

Hougie, C. 1971. Coagulation changes in healthy and sick Pacific salmon. *Adv. Exp. Med. Biol.* **22:** 89–102.

Hougie, C. 1977. Methods for estimating fibrinogen concentration. Pp. 1650–1651 in *Hematology,* 2nd ed., W. J. Williams, E. Beutler, A. J. Erslev, and R. W. Rundles, eds. McGraw Hill, New York.

Ingram, G. A., and J. B. Alexander. 1980. The immune response of brown trout *Salmo trutta* to lipopolysaccharide. *J. Fish Biol.* **16:** 181–197.

Katz, M., and M. Southward. 1950. The blood clotting time in spent silver salmon (*Onchorhynchus kisutch*). *Copeia* p. 150.

Klontz, G. W., and L. S. Smith. 1968. Methods of using fish as biological research subjects. Pp. 323–385 in *Methods of Animal Experimentation,* Vol. III, W. I. Gay, ed. Academic Press, New York.

Langdell, R. D., F. T. Bryan, and W. S. Gibson. 1965. Coagulation of catfish blood. *Proc. Soc. Exp. Med. Biol.* **118:** 439–441.

Levin, J., and E. Beck. 1966. A re-appraisal of the role of blood coagulation and platelets in the generalized Shwartzman phenomenon. *Thromb. Diath. Haemorrh.* **15:** 519–538.

Levin, J., and F. B. Bang. 1968. Clottable protein in Limulus: its localization and kinetics of its coagulation by endotoxin. *Thromb. Diath. Haemorrh.* **19:** 186–197.

McCarthy, D. H. 1976. Vibrio disease in eels. *J. Fish Biol.* **8:** 317–320.

MILLER, T. K. 1982. Coagulation changes in rainbow trout experimentally infected with *Yersinia ruckeri:* A possible role of endotoxin. *Abstr. Thirteenth Conf. Int. Assoc. Aquatic Anim. Med.* p. 16.

MILLER, T. K. 1983. *A Disseminated Intravascular Coagulation Syndrome in Rainbow Trout* (Salmo gairdneri) *During* Yersinia ruckeri *Infection.* Masters Thesis, University of Rhode Island, Kingston.

NEALSON, K. H. 1978. Isolation, identification and manipulation of luminous bacteria. *Methods Enzymol.* **57:** 153–165.

PATERSON, W. D., AND J. L. FRYER. 1974. Effect of temperature and antigen dose on the antibody response of juvenile coho salmon (*Oncorhynchus kisutch*) to *Aeromonas salmonicida* endotoxin. *J. Fish. Res. Board Can.* **31:** 1743–1749.

POL, J. M. A., AND J. M. V.D. BERG-BLOMMAERT. 1980. Pathogenesis of carp erythrodermatitis (CE). Pp. 120–132 in *Fish Diseases Third COPRAQ-session,* W. Ahne, ed. Springer Verlag, Berlin.

RAPAPORT, S. I., K. AAS, AND P. A. OWREN. 1954. The lipid inhibitor of brain: mechanism of its anticoagulant action and its comparison with the soybean inositol phosphatide inhibitor. *J. Lab. Clin. Med.* **44:** 364–373.

REYNOLDS, W. W., M. E. CASTERLIN, AND J. B. COVERT. 1977. Febrile responses of blue gill (*Lepomis macrochirus*) to bacterial pyrogens. *J. Thermal Biol.* **3:** 129–130.

RICHARDS, R. H., AND R. J. ROBERTS. 1978. Bacteriology of teleosts. P. 192 in *Fish Pathology,* R. J. Roberts, ed. Baillere Tindall, London.

SACK, G. H., JR., J. LEVIN, AND W. R. BELL. 1977. Trousseau's syndrome and other manifestations of chronic disseminated coagulopathy in patients with neoplasms: clinical, pathophysiologic, and therapeutic features. *Medicine (Baltimore)* **56:** 1–37.

STAUB, A. M. 1967. Preparation of cell wall antigens from gram negative bacteria. Pp. 28–34 in *Methods in Immunology and Immunochemistry Vol. I, Preparation of Antigens and Antibodies,* C. A. Williams and M. W. Chase, eds. Academic Press, New York.

THORSON, T. 1961. The partitioning of body water in Osteichthyes: Phylogenetic and ecological implications in aquatic vertebrates. *Biol. Bull.* **120:** 238–254.

UMBREIT, T. H., AND M. R. TRIPP. 1975. Characterization of the factors responsible for death of fish infected with *Vibrio anguillarum. Can. J. Microbiol.* **21:** 1272–1274.

VON ESCHEN, K. B., AND J. B. RUDBACH. 1974. Inactivation of endotoxin by serum. A phylogenetic study. *J. Infect. Dis.* **129:** 21–27.

WATSON, M. E., R. W. GUENTHER, AND R. D. ROYCE. 1956. Hematology of healthy and virus diseased sockeye salmon *Oncorhynchus nerka. Zoologica* **41:** 27–38.

WEDEMEYER, G. 1969. Pituitary activation by bacterial endotoxins in the rainbow trout. *J. Bacteriol.* **100:** 542–543.

WEDEMEYER, G., AND A. J. ROSS. 1968. Some metabolic effects of bacterial endotoxins in salmonid fishes. *J. Fish. Res. Board Can.* **31:** 1743–1749.